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The effect of endophytic fungi on growth and nickel accumulation in *Noccaea* hyperaccumulators

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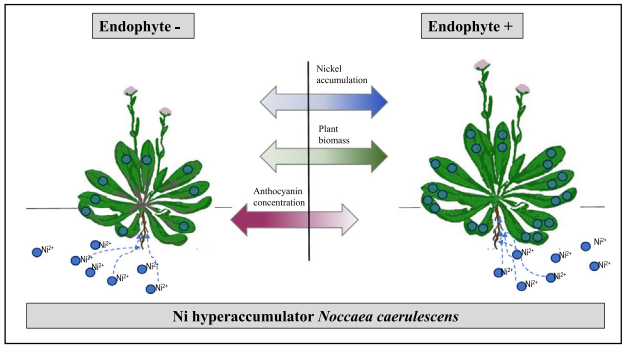
HIGHLIGHTS

- Brassicaceae Ni hyperaccumulators hosted multiple species of endophytic fungi.
- Selected fungi promoted host growth and increased Ni uptake.
- Specific genes were differently expressed in inoculated hyperaccumulating *Noccaea*.

GRAPHICAL ABSTRACT



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ABSTRACT

The role of endophytic fungi isolated from different populations of European Ni hyperaccumulators was investigated in regard to the microorganisms' ability to enhance the hyperaccumulation of Ni in *Noccaea caerulescens*. Effects of particular species of endophytic fungi on adaptation of *N. caerulescens* to excess Ni were tested by co-cultivation with single strains of the fungi. Seven of these had a positive effect on plant biomass production, whereas two of the tested species inhibited plant growth; biomass production of inoculated plants was significantly different compared to non-inoculated control. Inoculation with six fungal strains: *Embellisia thlaspis*, *Pyrenochaeta cava*, *Phomopsis columnaris*, *Plectosphaerella cucumerina*, *Cladosporium cladosporioides* and *Alternaria* sp. stimulated the plant to uptake and accumulate more Ni in both roots and shoots, compared to non-inoculated control. *P. columnaris* was isolated from all plant species sampled. Strains isolated from *Noccaea caerulescens* and *Noccaea goesingensis* increased Ni root and shoot accumulation of their native hosts (compared to non-inoculated control). Inoculation of different populations of *Noccaea* with *P. columnaris* of foreign origin did not cause its host to accumulate more Ni, with the exception of the Ni-unadapted ecotype of *N. goesingensis*. Inoculation with *P. columnaris* from *N. caerulescens* significantly improved Ni uptake, but the effect of the fungus was not as prominent as in the case of *N. caerulescens*. By comparing the transcriptomes of *N. caerulescens* and *N. goesingensis* from

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Flatz inoculated with *P. columnaris*, we showed that enhanced uptake and accumulation of Ni in the plants is accompanied by an upregulation of several genes mainly involved in plant stress protection and metal uptake and compartmentation.
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1. Introduction

Serpentine soils are naturally occurring metalliferous soils distributed worldwide (~1%) in the temperate and tropical regions and are usually enriched in metals Ni, Cr, and Co, deficient in essential nutrients (N, P, K, Mo, B) and organic matter, and have a low Ca to Mg ratio. Ni concentration can reach up to 3600 $mg \cdot kg^{-1}$ exceeding over 4-fold the concentrations found in soils globally (Sparks, 2003). Typically, serpentines are referred to as poor, shallow soils, with low water holding capacity, thus the plant cover of such habitats is limited to species with low trophic requirements and high metal tolerance (Kidd et al., 2018). Plants inhabiting such sites are either metallophytes that avoid toxic metal uptake into shoots (although they can accumulate high quantities of metals in their roots) or hyperaccumulators that accumulate Ni (usually also other metals such as Zn and Cd) in shoots (reviewed in Reeves et al., 2018). Hyperaccumulators can accumulate 100 times more Ni in their shoots than non-hyperaccumulating species (Brooks et al., 1977). To date 500 species of hyperaccumulators have been identified, a majority of which (ca. 90%) are Ni hyperaccumulators (reviewed in Cappa and Pilon-Smits, 2014). Ni hyperaccumulators were found in various plant families, suggesting independent evolution of hyperaccumulation; however, European representatives occur predominantly in the *Brassicaceae*: in tribes *Alysseae* and *Coluteocarpeae* (Cecchi et al., 2010; Krämer, 2010). In Europe, Ni hyperaccumulators occur most abundantly in the South of the continent. Their diversity follows a West to East gradient; two species were found in the Iberian peninsula, whereas app. 30 taxa were identified in South-East Europe, with the highest diversity in Greece and Albania (Al-Shehbaz, 2014; Reeves et al., 2018). In tropical and subtropical climate hyperaccumulators are also found in other plant families (Reeves, 2003). Several species yield significant biomass, which can be of great importance for Ni bioextraction and soil detoxification (van der Ent et al., 2013, 2017).

Regardless of their taxonomic affiliation, plants are inhabited by taxonomically diverse microbial communities referred to as the plant microbiome. These microorganisms include rhizospheric, endophytic bacteria and archaea (Moissl-Eichinger et al., 2018; Santoyo et al., 2016), rhizospheric, mycorrhizal fungi (Leyval et al., 1997) and endophytic fungi (Rodriguez et al., 2009) that are known to provide fitness benefits to their respective hosts. Several studies from the previous decades suggested that plants can reach their full hyperaccumulation capacity only in the presence of their indigenous microbiota (de Souza et al., 1999; Whiting et al., 2001). The majority of available studies concerning the role of microorganisms in Ni hyperaccumulation and potential application in Ni phytoextraction dealt with endophytic and rhizospheric bacteria (reviewed by Benizri and Kidd, 2018). Endophytic fungi have not been investigated in this manner, even though this group of microorganisms has been shown to facilitate vegetation in metal (Zn, Pb, Cd, Cu, Fe) enriched environments (Domka et al., 2019b; Rozpądek et al., 2018; Wężowicz et al., 2017). Additionally, the Brassicaceae do not associate with mycorrhiza, what indicates that endophytic fungi may be the dominant group of symbiotic fungi inhabiting plants from the Brassicaceae. Rhizospheric bacteria possess the ability to improve trace element solubility and thus availability in the rhizosphere. This has a positive impact on hyperaccumulation. Previously, Abou-Shanab et al. (2003) described increased acid production and metal solubility by rhizospheric bacteria from the hyperaccumulating *Odontarrhena muralis*. Besides bacteria, arbuscular mycorrhizal fungi (AMF) were found to facilitate growth of the Ni hyperaccumulator *Berkheya coddii* and other plants from the *Senecio* (*Asteraceae*) genus (Orłowska et al., 2011; Turnau and Mesjasz-Przybyłowicz, 2003) in serpentines by increasing nutrient (K, Fe, Zn, Mn, P, Ca) acquisition and optimizing distribution (Orłowska et al., 2013). Co and Ni uptake were inhibited in mycorrhizal plants which yielded significantly more biomass under metal toxicity. Very little is also known about the mechanistic aspect of the hyperaccumulator-symbiotic microorganism interaction; aside from the effect on nutrient acquisition and element distribution in symbiotic plants our knowledge about the mechanisms of improved adaptation is scarce.

In recent years hyperaccumulators have been extensively studied, due to their involvement in agromining, also called phytomining (Chaney et al., 2018): plants that can accumulate high quantities of trace metals are grown in metal/metalloid enriched soils and at the end of the vegetation period are harvested and burnt to produce metal/metalloid enriched ash or "bio-ore". According to available reports, this method of metal mining is considered commercially viable for Ni, Co, and Au (Chaney et al., 2018). Besides metal extraction from the soil, agromining can also provide multiple ecosystem services, such as: C sequestration, improve soil microorganism biodiversity, renewable biomass production, improved agricultural crop productivity and land restoration (Echevarria et al., 2015). Recently, several attempts have been made to improve the efficiency of phytomining by utilization of plant growth-promoting microorganisms (Cao et al., 2008; Durand et al., 2016). The primary aim of MAP (microorganism assisted phytoremediation) is to improve metal recovery rates by inoculating metal accumulating plants (including hyperaccumulators) with microorganisms, which can increase the plant metal accumulation capacity by: (i) improving plant biomass, and (ii) increasing plant uptake and bioaccumulation of metals (reviewed in Hrynkiewicz et al., 2018).

Symbiotic microorganisms including fungal endophytes facilitate plant adaptation to the environment, including environments enriched with toxic metals. Up till now, the main, known benefits conferred by rhizopheric and endophytic microorganisms are: i) improved root growth and root hair elongation; ii) increased nutrient availability and uptake; iii) upregulation of plant stress protection mechanisms; iv) optimization of metal uptake and distribution by the plant; v) biocontrol activity: competition for space and nutrients with pathogens and the production of antimicrobial compounds (reviewed in Benizri and Kidd, 2018; de Carvalho et al., 2020; Domka et al., 2019b; Kumar et al., 2019; Papik et al., 2020; Yan et al., 2019). Hyperaccumulating plants from the *Noccaea* genus are small, herbaceous and non-mycorrhizal; they are widely used as a model in studying the mechanisms of Zn and Ni hyperaccumulation. Another group of plants widely considered in phytoremediation are plants from the *Odontarrhena* genus that belong to the *Allysae* tribe. The advantage of using these plants is their relatively high biomass yield (Cerdeira-Pérez et al., 2019; Ghasemi et al., 2018). Their microbiome (as well as in other hyperaccumulating plants) however, has been poorly studied so far. The only available reports concern bacterial endophytes of *Noccaea caerulescens* and *N. goesingensis*, that were evaluated mainly utilizing cultivation dependent methods (Aboudrar et al., 2007; Idris et al., 2004, 2006; Visioli et al., 2014). There are no reports available concerning the community structure of *Noccaea* endophytic fungal symbionts. Very little is known about the role of these wide spread microorganisms in *Noccaeae* hyperaccumulation physiology.

The aims of this study were: i) to isolate and identify cultivable endophytic fungi from environments enriched in Ni; ii) to select species capable of improving plant Ni accumulation iii) to evaluate their role in *Noccaeae* ability to accumulate Ni in plant shoots; iv) to test the

specificity of the plant-microorganism interaction in terms of the ability of the microorganism to affect Ni hyperaccumulation of a non-native host; and v) to elucidate the mechanisms of microorganism dependent plant adaptation to serpentines, in terms of changes in plant transcriptome induced by symbiotic microorganisms potentially responsible for improved Ni accumulation in plant shoots.

2. Materials and methods

2.1. Plant collection

To establish a collection of culturable endophytic fungi from metal hyperaccumulators representatives of Ni-adapted populations of *Noccaea caerulescens* (J.Presl & C.Presl) F.K.Mey. from: Basadre and Bandeira (Spain), *N. goesingensis* (Halácsy) F.K.Mey. from Redlschlag (Austria) and *Odontarrhena serpyllifolia* (Desf.) Jord. & Fourr. from Bandeira (Spain) and non-toxic metal-adapted population *N. goesingensis* from Flatz (Austria) were collected. Detailed distribution of the plant populations is shown in Fig. 1. Three to seven plants from each population were collected in June for endophytic fungi isolation.

2.2. Isolation of endophytic fungi

Endophytic fungi were isolated from leaves, roots. Additionally, we were able to isolate endophytic fungi from seeds of the Pb-Zn and adapted Ni adapted (Bandeira). In the case of the other plant populations, due to the lack or limited number of seeds, the isolation of the endophytic fungi was not possible. Prior to microorganism isolation, plants were surface sterilized with 8% sodium hypochlorite for 5 min, followed by 96% ethanol for 1 min and 75% ethanol for 3 min and washed 5 times with sterile deionized water. A drop of water from the last rinsing was placed onto medium in order to confirm sterility. After surface sterilization, plants were cut into small segments (app. 3 × 3 mm) and placed onto Gel Gro (MP Biomedicals, USA) droplets supplemented with 0.03% $MgSO_4$ (Silvani et al., 2008) and antibiotics: ampicillin (40 mg·L^{-1}), streptomycin (40 mg·L^{-1}), and terramycin (20 mg·L^{-1}). Antibiotics were dissolved in sterile deionized water, filter sterilized using a 0.22 μm syringe filter and added to medium. For each plant 24 fragments (3 plates × 8 fragments) of each organ (leaf, root, and seed (if available)) were provided for endophyte isolation. Samples were incubated in the darkness at 27 °C and inspected every 1–2 days for 4 weeks. Cultures of emerging fungi were transferred onto potato dextrose agar (PDA) medium and incubated in darkness at 27 °C.

2.3. Identification of endophytic fungi

Pure cultures of endophytic fungi were identified based on morphological and anatomical features and according to the internal transcribed spacer (ITS) rDNA sequence. DNA was extracted with Genomic Mini AX Plant (A&A Biotechnology, PL) according to the manufacturers instruction. The ITS rDNA region was amplified with ITS1F (Gardes and Bruns, 1993) and ITS4 primers (White et al., 1990). Polymerase chain reaction (PCR) was performed in 25 μL reaction mixtures containing 1–4 ng of DNA matrix; 9.5 μL of nuclease-free water; 12.5 μL of Maxima Hot Start Green PCR Master Mix (Thermo Scientific), and 1 μL of each of

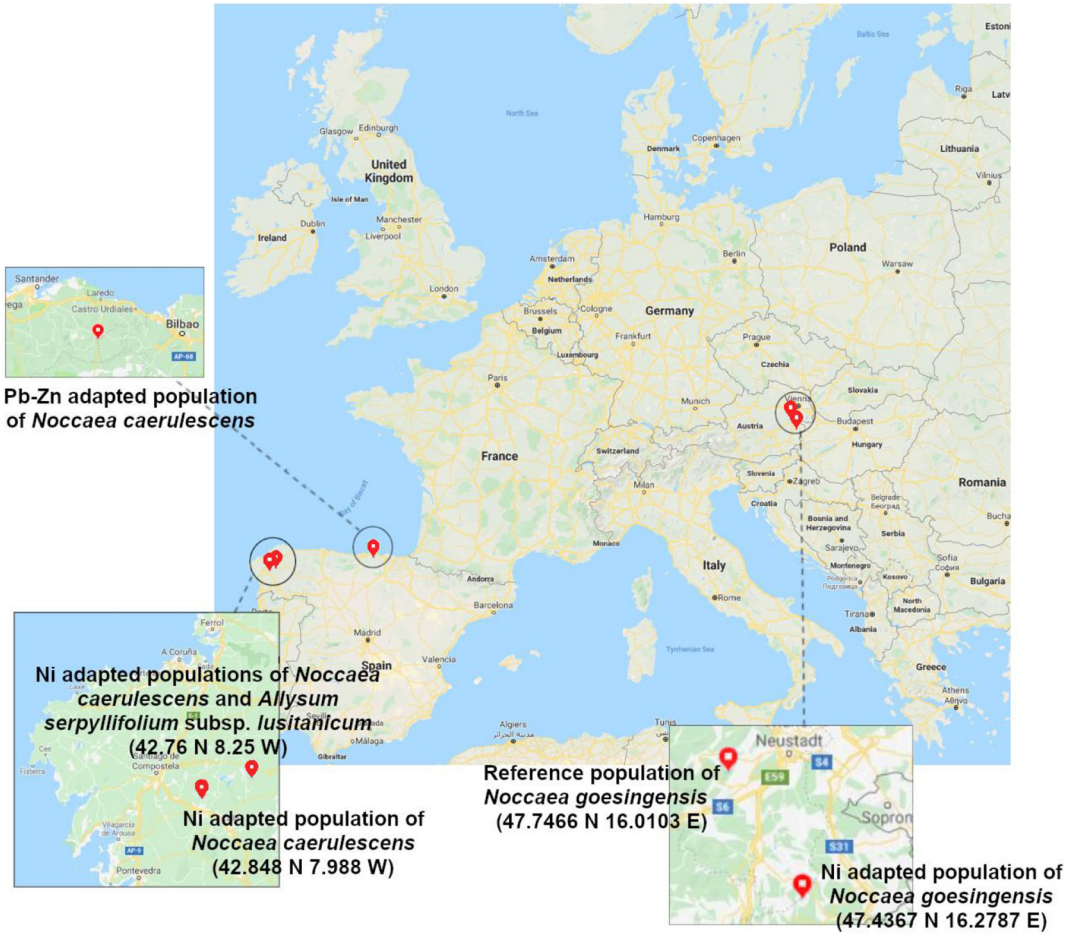


Fig. 1. Location of the populations of *Noccaea caerulescens*, *N. goesingensis* and *Odontarrhena serpyllifolia*. The sites include two *N. caerulescens* populations and one *O. serpyllifolia* population from serpentine outcrops in Spain (Basadre, Bandeira), one *N. caerulescens* population from Zn-Pb enriched soils in northern Spain (Basque-Cantabrian Basin), one *N. goesingensis* population from serpentine soils in eastern Austria (Redlschlag) and one *N. goesingensis* population from non-toxic metal enriched soils in Austria (Flatz).

the primers at 10 pmol concentration for each sample. PCR conditions included: 1) initial denaturation at 95 °C for 3 min; 2) 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 45 s; 3) final elongation at 72 °C for 5 min. The presence of PCR products was visualized in 1.5% agarose gel stained with GelRed (Biotium). Isopropanol and 0.3 M sodium acetate precipitation were used for purification of PCR products (http://openwetware.org/wiki/Isopropanol_Precipitation_for_PCR_Purification). The PCR products were sequenced by Macrogen Laboratory (NL). The ITS4 primer was used for reading sequences. The sequences were edited with Chromas software (www.technelysium.com.au) and subsequently compared with sequences published in the NCBI database (www.ncbi.nlm.nih.gov) by BLASTn algorithm. Fungi species were identified if at least 98% sequence similarity of ITS region matched reference sequences. Sequence data were deposited in the NCBI database under accession number MT357198 - MT357255.

2.4. Plant growth response tests

To test the role of endophytic fungi in the adaptation of *Noccaea* to Ni toxicity, *N. caerulescens* seedlings were inoculated with a single strain of twenty-three selected fungal endophytes (single inoculation experiments). This included endophytic fungi isolated in our previous study from Pb-Zn-adapted population of *N. caerulescens* from Basque Country (Spain): *Embellisia thlaspis*, *Phialocephala fortinii*, *Plectosphaerella cucumerina*, *Neocucurbitaria cava*, *Amycosphaerella africana*, *Septoria* sp., *Alternaria* sp. and *Periconia byssoides*. For a single plant – fungus consortium at least thirty *N. caerulescens* seedlings were inoculated (23 fungi × 30 seedlings = 690 seedlings). The experiment was performed in 5 series. This was necessary to prevent cross contamination of the different plant-fungus consortia.

Plant preparation, vegetation condition details: plant seeds were surface sterilized in 8% sodium hypochlorite for 5 min, followed by 96% ethanol for 1 min and 75% ethanol for 3 min and washed 5 times with sterile deionized water and then germinated in a substrate composed of a mixture of sterile sand and vermiculite (1:2; v:v). The germination was conducted at 4 °C in darkness for 2 days, followed by a 16 h photoperiod in 140 μM at 21/17 °C and humidity of 60/70% for 12 days. Afterwards, seedlings were transferred into pots with the same substrate and after 5 days inoculated with the endophytic fungi. Each seedling ($n = 30$) was inoculated with 3 mL of the liquid inoculum containing fungal mycelium. Endophytic fungi were cultivated on malt extract medium (2%) at 24 °C for 5 days. The mycelium was filtered, washed in sterile deionized water, homogenized and suspended in 50 mL sterile deionized water. The plants were irrigated twice a week with 6 mL sterile deionized water or Hoagland solution (2 mM $MgSO_4$ $7H_2O$, 0.8 mM $Ca(NO_3)_2$, 2.5 mM KNO_3, 0.1 mM K_2HPO_4, 20 μM FeEDDHA, 10 μM H_3BO_3, 2 μM $MnCl_2$, 1 μM $ZnSO_4$, 0.5 μM $CuSO_4$ $5H_2O$, 0.2 μM Na_2MoO_4). After 14 days the plants were irrigated with Hoagland solution supplemented with 150 μM $NiSO_4$ $5H_2O$. Plants were grown in a vegetation chamber at 21/17 °C, 16 h photoperiod in 190 μM s^{-1} m^2. Control, not inoculated plants and plants grown with a particular fungal strain were cultivated in separate chambers. Six weeks after inoculation the plants were harvested and evaluated for the fresh and dry weight. For dry weight determination, plants were dried at 80 °C for 48 h.

2.5. Verification of plant colonization by endophytic fungi

Verification of plant colonization by endophytic fungi was carried out on plant material derived from in-vitro cultures and from pot cultures as well. Plant seeds were sterilized as described in Section 2.4 and sown onto Petri dishes with sterile Murashige and Skoog medium (1/4 strength) supplemented with 0.75% sucrose and also to substrate composed of a mixture of sterile sand and vermiculite (1:2; v:v). After incubation at 4 °C for 48 h, plates were transferred to a growth chamber (Panasonic, Japan; MLR-352H-PE, 21/17 °C, continuous illumination 140 μmol m^2s^{-1}, 16-h photoperiod). After 7 days, plants were transferred to square Petri dishes with Strullu-Romand phyto-agar (Duchefa, NL) supplemented with 150 μM $NiSO_4$ $5H_2O$ and inoculated with the endophytic fungi by placing 3 mm agar plugs of the mycelium on the top of the medium at a distance of 1 cm from the roots. Plants not inoculated with the fungi served as control. After 14 days plants were harvested and stained. The handling with plants in pot cultures was analogous as described in Section 2.4. Six weeks after inoculation plants were harvested and stained. Plants from in-vitro and pot cultures ($N = 5$) were stained with trypan blue and Sudan IV, according to modified procedure of Barrow (2003). As a modification a vacuum steamer was used instead of autoclave. The colonization was verified by observation with light microscope.

2.6. Nickel concentration

To test the role of endophytic fungi in *Noccaea* Ni accumulation, Ni concentration was measured in *N. caerulescens* seedlings inoculated with 23 strains of endophytic fungi in single inoculation experiments. Non-inoculated seedlings served as control. Approximately 50 mg of plant samples (root and shoot separately; 8–10 plants per sample) were weighed to analytical accuracy and transferred into teflon autoclave, then 5.00 mL of 65% HNO_3 (Argenta, PL) were added to each tube and the samples were predigested at room temperature for 1 h. Subsequently, 2.00 mL of 30% hydrogen peroxide (Sigma Aldrich, USA) were added and the digestion was carried out for the next 30 min. Microwave digestion was carried out for 35 min (temp profile: step 1 – ramp 5 °C/min, time – 5 min, temp: 145 °C; step 2 – ramp 3 °C/min, time – 10 min, temp: 190 °C; step 3 – ramp 10 °C/min, time – 1 min, temp: 75 °C). After cooling to room temperature, the solution was transferred into a 25 mL volumetric flask and made up with deionized water. The blank samples were processed simultaneously according to the same analytical procedure. Nickel was measured by using Spectrometry (Graphite Furnace Atomic Absorption Spectroscopy [GF-AAS], equipped with an auto-sampler [Thermo Scientific, iC3000]). The external standard calibration method was applied using AAS standard solutions (Sigma Aldrich). Trace grade chemicals were used for the determination of Ni concentration. The analysis was performed in 3 replicates per fungal strain.

2.7. Anthocyanin concentration

To evaluate the stress protective role of endophytic fungi, anthocyanin concentration in leaves of inoculated plants was measured. Anthocyanin concentration was not measured in all plant-endophyte consortia used in the previous screening. Only four endophytic fungi (*Phomopsis columnaris*, *Plectosphaerella cucumerina*, *Embellisia thlaspis*, and *Amycosphaerella africana*), selected according to their ability to improve Ni accumulation in the shoots, were used in this experiment. To analyse anthocyanin concentration in *N. caerulescens* shoots an independent experiment was setup as described in Section 2.4. For control, non-inoculated plants treated with 150 μM $NiSO_4 \cdot 5H_2O$ and not treated with Ni were used. Anthocyanin concentration was determined spectrophotometrically according to the method described by Fukumoto and Mazza (2000). Leaf tissue (5–6 plants per sample) was homogenized in liquid nitrogen (LN_2). Extraction from fine-powdered leaf tissue was performed in ice-chilled 80% methanol with glass beads in a temperature-controlled homogenizer (Tissuelyser, Qiagen, USA). Before readings, the reaction mixture consisting of 0.25 mL sample, 0.25 mL of 0.1% HCl in 95% ethanol and 4.50 mL of 2% HCl was incubated at room temperature in darkness for 30 min. For the analysis, 1 mL of the reaction mixture was used. Absorbance readings at 520 nm were interpolated against the calibration curve prepared with cyanidin (Sigma, USA) as a standard.

2.8. Fungal tolerance to toxic metals

Phomopsis columnaris was one of the fungal strains which allowed its host to accumulate more Ni and provided protection against metal toxicity. It was also isolated from all plants from serpentines used in this study. Most important, however, inoculation with this fungus did not affect biomass production. This feature of *P. columnaris* made it an optimal candidate to investigate the fungi dependent mechanisms of Ni hyperaccumulation in *N. caerulescens* independent of fungi effect on plant growth. To test *P. columnaris* tolerance to Ni toxicity, the strain isolated from *N. caerulescens* was grown on PDA supplemented with a gradient of Ni ($NiSO_4$ ranged from 0 to 3000 μM), Zn ($Zn(NO_3)_2 \cdot 6H_2O$ ranged from 0 to 4000 μM) and Cd ($Cd(NO_3)_2 \cdot 4H_2O$ ranged from 0 to 4000 μM). Metal precursors were added by a sterile 0.22 μm syringe filter to cooled (app. 50 °C) autoclaved medium. Three replicates were prepared of each metal concentration. The medium was inoculated with agar plug of the mycelium and incubated at 26 °C in darkness for 3 days. Fungus growth was determined by mycelium area with ImageJ (NIH, USA) software (under GNU General Public License). The fungal tolerance index (TI) was calculated as the ratio of the surface area of the fungal colony exposed to particular Ni concentration versus control plate. At the same time, a non-Ni adapted *P. columnaris* strain (isolated from *Arabidopsis arenosa* inhabiting a Pb-Zn mine dump) was subject to the same treatment. This experimental setup allowed us to investigate: 1) Ni tolerance of the fungus isolated from *N. caerulescens*, 2) the specificity of mechanisms involved in fungal Ni tolerance.

2.9. Cross-inoculation of different species of hyperaccumulators with different strains of P. columnaris

Three different strains of *Phomopsis columnaris* were isolated from three serpentines investigated in this study. To test the specificity of the *P. columnaris* - Ni hyperaccumulators interaction, in terms of the ability of the plant to improve Ni accumulation, we measured root and shoot concentration of Ni in two different Ni-adapted plant populations: *N. caerulescens* and *N. goesingensis* (from Redlschlag, Austria) with different *P. columnaris* strains isolated from Ni-adapted populations of *N. caerulescens*, *N. goesingensis* (from Redlschlag, Austria) and *O. serpyllifolium*. Additionally, we inoculated with the same endophytes a Ni-unadapted ecotype of *N. goesingensis* (from Flatz, Austria) to see whether we can induce hyperaccumulation with an endophytic fungus in a plant that was not adapted to metal toxicity. In cross-inoculation experiments, each plant population was inoculated with a single fungal strain. Ni concentration was measured as described above (Section 2.5). To test plant - *P. columnaris* strain specificity in regard to the fungi's ability to affect Ni uptake by the plants an independent experiment was setup as described in Section 2.4.

2.10. RNA sequencing

N. caerulescens and *N. goesingensis* (Flatz) inoculated with *P. columnaris* isolated from *N. caerulescens* were prepared as described in Section 2.4. Total RNA was extracted from frozen ground roots in liquid nitrogen (from 5 to 7 plants per sample) with the Total RNA Mini Kit (Bio-Rad, US). RNA purity and quantity were determined by Biospec-Nano (Shimadzu, JP). The integrity of RNA was assessed with the Agilent 2100 Bioanalyzer (USA) and RNA 6000 Nano Kit (Agilent, DE).

Whole transcriptome libraries were prepared using Ion Total RNA-seq Kit v2 (ThermoFisher Scientific, USA). 1000 ng of purified (DNAase treated) total RNA was used for poly(A) RNA selection performed with Dynabeads mRNA DIRECT Micro Kit (ThermoFisher Scientific, USA) following manufacturer's protocol. The selected poly(A) RNA was subsequently fragmented with RNase III and purified. The quality of fragmented RNA was analyzed with Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and the RNA 6000 Pico Kit (Agilent Technologies, USA). Subsequently, the RNA was hybridized, ligated and reverse transcription was performed using the Ion Total RNA-seq Kit v2. After purification, the cDNA was amplified and barcoded with the Ion Xpress RNA-Seq Barcode 1–16 Kit (ThermoFisher Scientific, USA) and purified. The yield and size distribution of amplified DNA was assessed with High Sensitivity DNA Kit using the 2100 Bioanalyzer (Agilent Technologies, USA). Finally, all of the prepared libraries were diluted to equimolar concentrations (100 pM) and pooled to sets of 6 samples. In the next step, template-positive Ion PI Ion Sphere Particles (ISPs) with 200 base-pair average insert libraries for sequencing were prepared and enriched using Ion PI Hi-Q OT2 200 Kit (ThermoFisher Scientific, USA) and Ion OneTouch 2 System (ThermoFisher Scientific, USA) following the protocol provided by the manufacturer. Sequencing was performed using reagents and materials included in Ion PI Hi-Q Sequencing 200 Kit (ThermoFisher Scientific, USA) and Ion PI Chip Kit v3 (ThermoFisher Scientific, USA) on the Ion Proton System (ThermoFisher Scientific, USA) according to standard manufacturer's protocol. Raw signal produced by the Ion Torrent Sequencer were converted to calls bases with associated per-base quality values using Torrent Server with Torrent Suite software. Reads were stored in standard FASTQ files and the quality control was performed using FastQC software (ver. 0.11.5). Estimated abundance of transcripts was obtained using Salmon tool (ver. 0.7.2). Finally, the differential expression analysis was performed using edgeR package (ver. 3.20.9) for R. To identify genes differently expressed in the hyperaccumulating *Noccaeae* plants after inoculation with the endophytic fungus we compared the response of the transcriptome of endophyte-inoculated (E+) *Noccaea caerulescens* grown in substrate supplemented with Ni (Ni+) with *N. caerulescens* E- Ni- and *N. goesingensis* (Flatz ecotype) E+ Ni+ vs *N. goesingensis* (Flatz ecotype) E- Ni+. These two species were selected to the transcriptomic analysis due to differences in their response to inoculation with the fungus in terms of Ni uptake and accumulation in the shoot. A twofold up or down regulation cut-off and a corrected *P*-value cut-off of 0.01 was applied to selected differentially regulated genes in plants inoculated with the endophytic fungus compared to non-inoculated plants. Differently regulated gene functional classification was performed by DAVID (Huang et al., 2009a, 2009b).

2.11. Statistical analysis

Statistical comparisons were performed using Statistica 12 (StatSoft) and were considered significant at $P \leq 0.05$. Data normal distribution and variance homogeneity were assessed with Shapiro-Wilk's and Levene's tests, respectively. If necessary, data were normalised with a log10 transformation. Differences were tested by analysis of variance (ANOVA) followed by the Tuckey's or Dunnett post-hoc tests.

3. Results

3.1. Fungal endophytes isolated from hyperaccumulating plants

Eighty seven fungal strains were isolated from *Noccaea* and *Odontarrhena* plants. According to the sequences of the ITS rRNA region, a total of 40 endophytic fungal taxa inhabited hyperaccumulating plants (Table 1, Supplementary Table 1). A mean number of 2.9 strains and 1.3 species per plant were isolated. The majority (95%) of the fungi that we isolated belonged to the Ascomycota (classes: Dothideomycetes, Sordariomycetes, Eurotiomycetes and Leotiomycetes). From the Ascomycota, the Dothideomycetes and the Sordariomycetes classes were the most highly represented (17 and 11 species, resceptively). Two representatives of the Basidiomycota (*Rhizoctonia solani* and *Campanella caesia)* were identified. The most frequent endophytes isolated were *Phomopsis columnaris* and *Embellisia thlaspis*. *P. columnaris* was isolated from plants collected in all Ni-adapted populations: *N. caerulescens*, *N. goesingensis* and *O. serpyllifolia*. *E. thlaspis* was found in both species of the *Noccaea* genus.

3.2. Plant biomass production upon inoculation with endophytic fungi

Strains of seven species of endophytic fungi had a positive effect on plant biomass production. The following improved plant fresh weight: *Plectosphaerella cucumerina*, *Embellisia thlaspis* (1), *Septoria* sp., *Alternaria* sp., *Cladosporium cladosporioides* (1), *Cadophora luteo-olivacea* and *Phoma herbarum*. Biomass improvement ranged from 1.3-fold to 2.2-fold in comparison to non-inoculated plants (Fig. 2). Plant dry weight was improved by five of the above-mentioned species (*Plectosphaerella cucumerina*, *Embellisia thlaspis* (1), *Septoria* sp., *Cadophora luteo-olivacea and Phoma herbarum*) and by *Penicillium brasilianum* and *Elaphocordyceps subsessilis* (Fig. 2). Inoculation with *Fusarium redolens* negatively affected plant fresh and dry weight production, whereas *Neocucurbitaria cava* significantly decreased plant dry weight. All other fungi did not affect biomass production by the plant (Fig. 2).

3.3. Verification of plant colonization by endophytic fungi

In planta presence of all of the fungi strains tested was verified positively (Table 2). All the plants collected for fungal staining were fully green and without any symptoms of parasitic fungal growth. In presence and in absence of Ni the mycelium was visible entering the apical part of young roots (Fig. 3A). Also the root hairs which were often irregular or branched were strongly colonized by fungal mycelium. This was the best visible when the freshly formed root hairs of up to 100 μm length were observed (Fig. 3B). Less often the mycelium was visible in longer root hairs (Fig. 3C–D) but at this stage more mycelium was visible in the root cortex (Fig. 3C, E), within periendodermal layer and vascular tissue (Fig. 3F). In case of *P. columnaris* also "Hartig net" like structures were observed close to periendodermal layer (Fig. 3G). The mycelium entered via vascular tissue and finally was found in leaves.

Table 1
Microbial composition of *Noccaea caerulescens*, *N. goesingensis* and *Odontarrhena serpyllifolia* isolated endophytic mycobiota. Taxonomic assignment is shown at the phylum or species level. Detailed description of identified strains is presented in Supplementary Table 1.

	N. caerulescens			*N. goesingensis*		*O. serpyllifolia*
	Basque Country	Bandeira	Basadre	Redlschlag	Flatz	Basadre
Alternaria infectoria						
Alternaria sp.						
Amycosphaerella africana						
Aspergillus fischeri						
Aspergillus sp.						
Botryosphaeria dothidea						
Byssochlamys verrucosa						
Cadophora luteo-olivacea						
Campanella caesia						
Cladosporium cladosporioides						
Cladosporium sp.						
Colletotrichum spaethianum						
Cyphellophora sp.						
Darksidea sp.						
Diaporthe hongkongensis						
Diaporthe novem						
Diaporthe sp.						
Elaphocordyceps subsessilis						
Embellisia thlaspis						
Fusarium avenaceum						
Fusarium redolens						
Fusarium sp.						
Lachnum virgineum						
Lophiostoma sp.						
Myceliophthora verrucosa						
Neocucurbitaria cava						
Paraphoma radicina						
Penicillium brasilianum						
Penicillium skrjabinii						
Penicillium sp.						
Periconia byssoides						
Phialocephala fortinii						
Phoma exigua						
Phoma herbarum						
Phomopsis columnaris						
Plectosphaerella cucumerina						
Pleosporales sp.						
Rhizoctonia solani						
Septoria sp.						
Simplicillium lamellicola						
Unidentified						

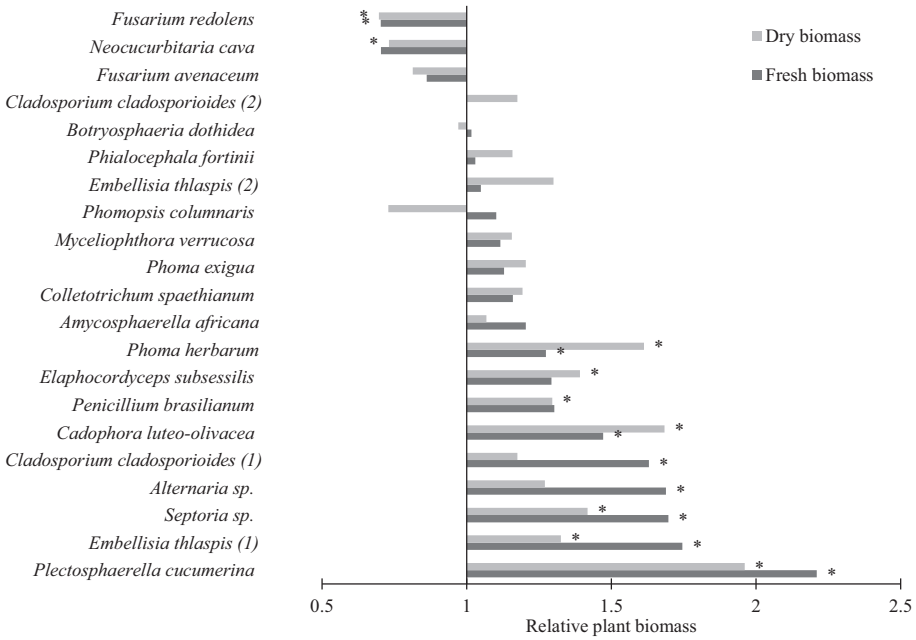


Fig. 2. Relative fresh and dry weight of *Noccaea caerulescens* plants inoculated with endophytic fungi. Weight of plants is presented in relation to uninoculated control plants. For each strain of endophytic fungi, thirty *N. caerulescens* seedlings were inoculated in single inoculation experiments. Plants were grown in sand and vermiculite (1:2, v:v), irrigated with Hoagland solution supplemented with 150 μM $NiSO_4$. Eight week old plants were harvested for analysis. Statistical significance was evaluated with the Dunnett test at $p \leq 0.05$ ($N = 30$).

The hyphae were often visible within leaf cortical cells around vascular tissue (Fig. 3H) and also between leaf epidermal cells (Fig. 3J). *P. columnaris* was successfully reisolated from *N. caerulescens* roots. The presence of the fungus in plant leaves was not confirmed by reisolation.

3.4. Ni accumulation

The following endophytic species: *E. thlaspis* (1), *N. cava*, *P. columnaris*, *P. cucumerina*, *C. cladosporioides* and *Alternaria* sp. increased significantly Ni accumulation in both roots and shoots of *N. caerulescens*. Only one species, *Amycosphaerella africana*, increased Ni accumulation in the shoots of *N. caerulescens* without affecting Ni accumulation in the roots. Two species: *Myceliophthora verrucosa* and *C. cladosporioides* (2) improved Ni uptake into plant roots, without affecting accumulation in the shoots. Ni concentration was increased from 1.4 to 1.6-fold in comparison to non-inoculated plants (Fig. 4, Supplementary Table 2).

3.5. Anthocyanin

Anthocyanin concentration in leaves of *N. caerulescens* grown in the substrate supplemented with Ni reached 6.7 mg·g FW^{-1} and was significantly higher than in control plants (grown in medium without Ni; 5.4 mg·g FW^{-1}). After inoculation with *Phomopsis columnaris*, *Plectosphaerella cucumerina*, *Embellisia thlaspis* (1) and *Amycosphaerella africana* (in single inoculation experiments), plants, grown in Ni-supplemented medium, accumulated significantly less anthocyanins. *Noccaea* inoculated with *Phomopsis columnaris* accumulated 3.2 mg·g FW^{-1}, with *Plectosphaerella cucumerina* 3.4 mg·g FW^{-1}, with *Embellisia thlaspis* 4.4 mg·g FW^{-1} and 3.9 mg·g FW^{-1} when inoculated with *Amycosphaerella africana* (Fig. 5).

Table 2
In planta presence of selected endophytic fungi.

Fungus strain	In vitro		Pot cultures	
	Root	Shoot	Root	Shoot
Cladosporium cladosporioides	+	+	+	+
Phomopsis columnaris	+	+	+	−
Embellisia thlaspis	+	+	−	+
Septoria sp.	+	−	−	+
Alternaria sp.	+	+	+	+

3.6. Toxic metal tolerance of Phomopsis columnaris

The tolerance index (TI) to Ni of *P. columnaris* from the serpentine was unchanged up to 1500 μM of Ni and ranged from 0.90 to 1.06. Significant TI decrease started at 2000 μM of Ni (TI = 0.80) and reached the lowest value (TI = 0.13) at 3000 μM (Fig. 6A–B). Cd imposed a significant change in *P. columnaris* mycelium growth at concentrations of 15 μM and higher (Fig. 6C), whereas treatment with Zn concentrations of 2500 μM and higher resulted in a gradual inhibition of mycelium growth (Fig. 6D). The tolerance index of the strain isolated from the Pb-Zn mine dump was not changed by any of the metal treatments (Fig. 6A–D).

3.7. Cross-inoculation of different species of hyperaccumulators with different strains of P. columnaris

N. goesingensis Redlschlag accumulated significantly more Ni in its shoots after inoculation with its native *P. columnaris*. Similarly, *N. caerulescens* accumulated more Ni with its shoots, when the plants were inoculated with its native strain *P. columnaris*. Non-native fungi (not isolated from the host plant) did not affect Ni accumulation in shoots and roots of serpentine populations of *Noccaea caerulescens* and *N. goesingensis* Redlschlag; they were, however, able to improve Ni accumulation in roots of non-serpentine *N. goesingensis* from Flatz but were not able to improve metal accumulation in the plant shoots (Fig. 7). Interestingly, all of the plants interacted with the fungus. We were able to re-isolate the fungus from all *Noccaea* plants tested, indicating that it interacted with all of the examined plants.

3.8. Gene expression

In *N. caerulescens* the expression of 472 genes was upregulated in E + plants compared to *E*-. Out of this gene set, the expression of 58

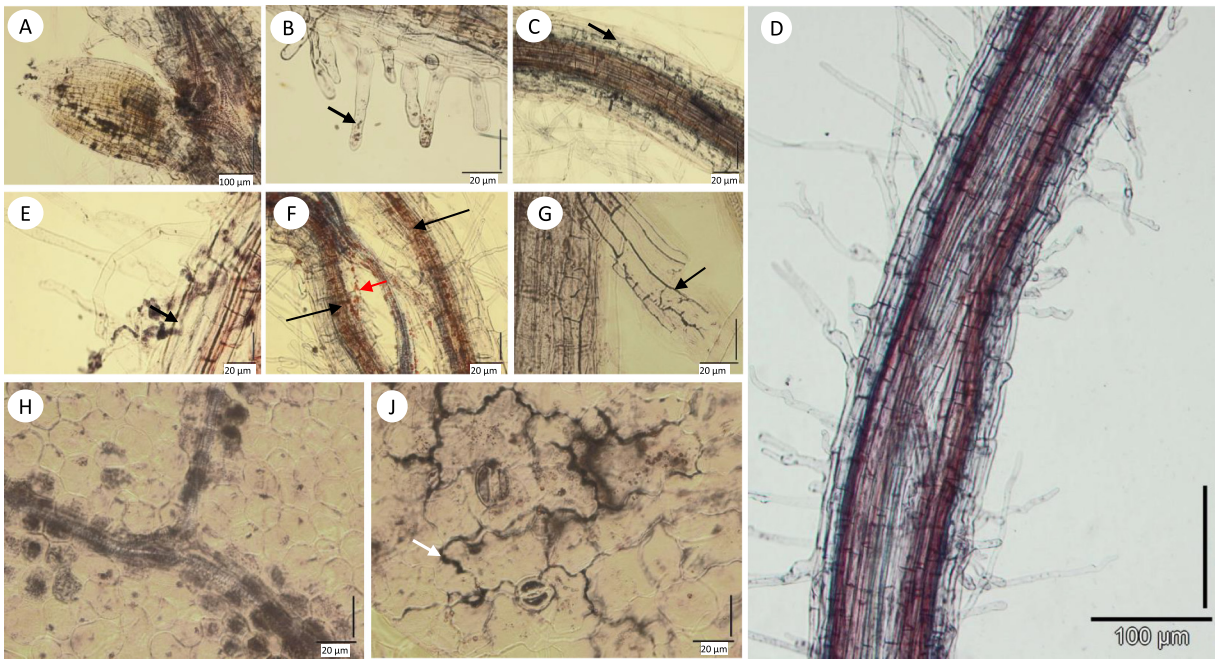


Fig. 3. Colonization of *Noccaea goesingensis* by endophytic fungus *Phomopsis columnaris*: (**A**) mycelium entering the apical part of young roots; (**B**) young root hairs with visible fungal hyphae (white arrow) with lipids (stained red) inside (**C**) blue stained fungal mycelium (arrow) within cortical layer; (**D**) branched root hair of plants in presence of endophytic fungi and Ni; (**E**) blue stained mycelium (arrow) visible in the root cortex and root hairs; (**F**) red stained lipids in mycelium (red arrow) within periendodermal layer (black arrow) and vascular tissue; (**G**) "Hartig-like" net structures (arrow) observed close to periendodermal layer; (**H-J**) hyphae visible within leaf cells around vascular tissue (**H**) and between leaf epidermal cells (**J**).

genes was upregulated in both plant species: *N. carulescens* E+ Ni+ and *N. goesingensis* (Flatz) E+ Ni+, 7 was down-regulated and 407 unaffected in *N. goesingesis* (Flatz). In *N. goesingensis* (Flatz) the expression a total of 607 genes was upregulated. The expression of 532 of these genes was unaltered in *N. caerulescens* and 17 was down regulated. Out of the set of *N. caerulescens* genes with down regulated expression 14 genes were common for both plant species, the expression of 17 was upregulated and 326 not affected in *N. goesingensis* (Flatz). In *N. goesingensis* (Flatz) the expression of 202 genes was down regulated. The expression of 181 genes from this gene set was not affected in *N. caerulescens* and the expression of 7 was upregulated. A graphical representation of differently genes expressed in *N. caerulescens* E+ Ni + vs *N. caerulescens* E- Ni+ and *N. goesingensis* (Flatz) E+ Ni+ vs *N. goesingensis* (Flatz) E- Ni+ and the relationships in these two data sets are shown in Fig. 8A. To select genes potentially involved in the accumulation phenotype of E+ *N. caerulescens* functional annotation/categorization of genes with up and down regulated expression, excluding genes with up and down regulated expression respectively in both species was performed. Genes with upregulated expression were classified into 47 distinct functional categories in *N. caerulescens* and 26 in *N. goesingensis* (Flatz). The majority of the GO terms enriched in *N. caerulescens* E+ and *N. goesingensis* (Flatz) E+ were involved in plants defense response; response to different biotic and abiotic stress factors and regulation of hormone (ethylene, jasmonic acid, salicylic acid, abscisic acid) and hydrogen peroxide related metabolism and biosynthesis (31 GO terms in *N. caerulescens* and 14 in *N. goesingensis*

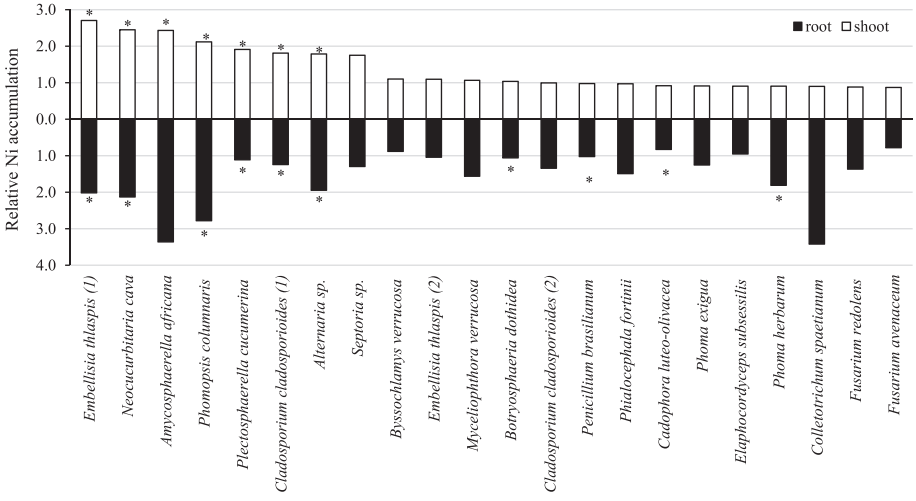


Fig. 4. Relative Ni accumulation in shoots and roots of *Noccaea caerulescens* upon endophytic fungi inoculation. For each strain of endophytic fungi, thirty *N. caerulescens* seedlings were inoculated in single inoculation experiments. Plants were grown in sand and vermiculite (1:2, v:v), irrigated with Hoagland solution supplemented with 150 µM $NiSO_4$. Eight week old plants were harvested for analysis. Ni concentration is presented in relation to the values measured in uninoculated control plants, statistical significance was evaluated with the Dunnett test at $p \leq 0.05$ (N = 3).

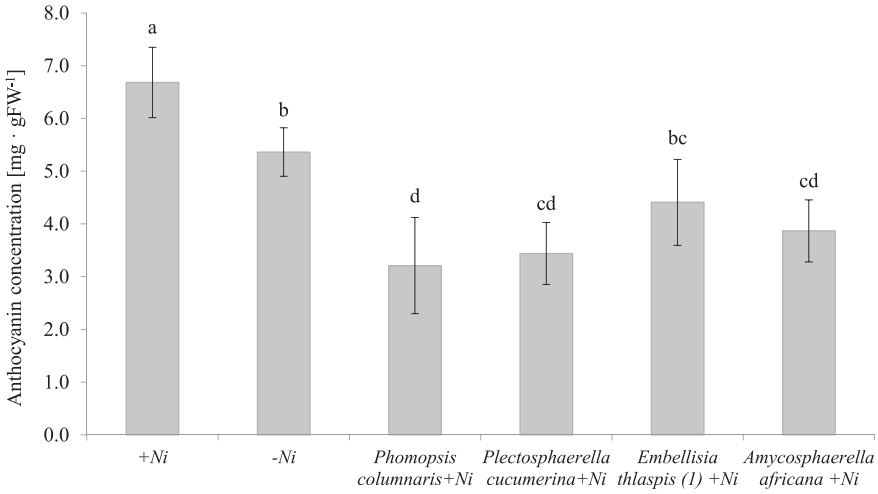


Fig. 5. Effect of fungal inoculation on anthocyanins accumulation in shoots of *N. caerulescens* treated with Ni. For each strain of endophytic fungi, thirty *N. caerulescens* seedlings were inoculated in single inoculation experiments. Plants were grown in sand and vermiculite (1:2, v:v), irrigated with Hoagland solution supplemented with 150 μM $NiSO_4$. Eight week old plants were harvested for analysis. Bars indicate ± SD, statistical significance was tested by the one-way analysis of variance (ANOVA) followed by the Fisher's LSD post-hoc test at $p \leq 0.05$ ($N = 5$).

(Flatz)). Additionally, in both plants, the expression of genes involved in gene expression (regulation of transcription, methylation, five and four GO terms respectively) camalexin biosynthesis and in vasculature development (a single GO term in both species) were upregulated. The expression of genes classified in GO terms: seed coat development, fatty acid biosynthetic process, cellulose catabolic process, aromatic compound biosynthetic process, zinc II ion transmembrane transport (Fig. 8B), shikimate metabolic process were upregulated in *N. caerulescens* but not in *N. goesingensis* (Flatz). On the contrary, the expression of genes classified in GO terms: glutamine metabolic process, iron-sulfur cluster assembly, phosphatidylinositol dephosphorylation, proline catabolic process to glutamate, regulation of response to red or far red light, calcium ion transmembrane transport, inositol catabolic process were upregulated in *N. goesingensis* (Flatz) but not in *N. caerulescens*. The majority of the genes with downregulated expression in both plants were classified into GO term "oxidation-reduction process" (32 and 17 genes in *N. caerulescens* and respectively *N. goesingensis* (Flatz)), defense response, particularly to biotic stress factors such as insect and bacteria including

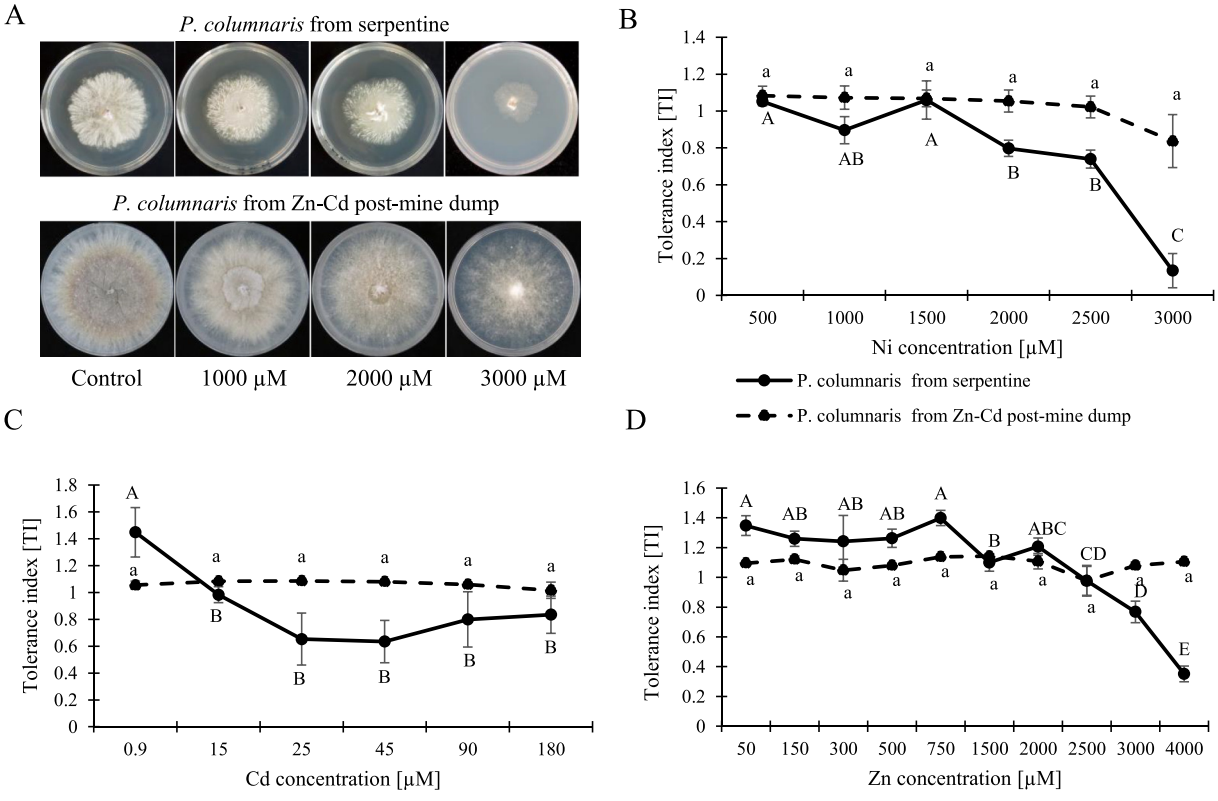


Fig. 6. Effect of different Ni, Cd and Zn concentration on *Phomopsis columnaris* growth. (**A**) *P. columnaris* strains isolated from plants collected from serpentine and Zn-Cd post-mine dump growing on media supplemented with Ni; Tolerance index (TI) calculated for *P. columnaris* strains isolated from plants collected from serpentine and Zn-Cd post-mine dump growing on media supplemented with Ni (**B**) Cd (**C**) and Zn (**D**); error bars show ± SD, different letters above the bars indicate statistically significant differences at $p \leq 0.05$ ($N = 3$), lowercase letters correspond to data for the serpentine strain and capital letters correspond to data for Zn-Cd post-mine dump strain.

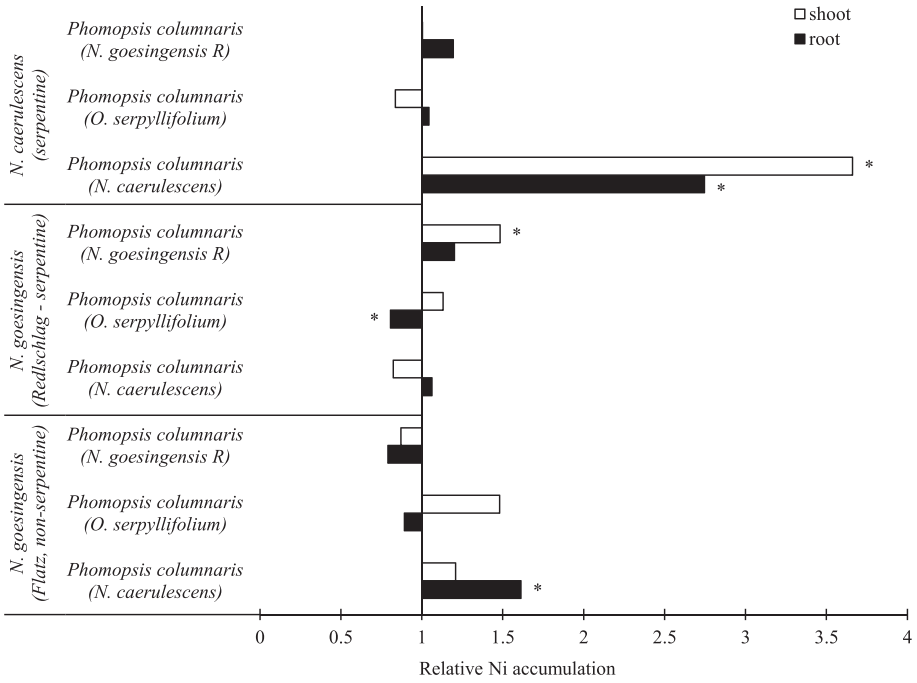


Fig. 7. The effect of three different isolates of *P. columnaris* on Ni accumulation in shoots and roots of *N. caerulescens* and two *N. goesingensis* populations. *N. caerulescens* and plants from Ni adapted Redlschlag population and reference Flatz population were inoculated with *P. columnaris* strains isolated from *N. caerulescens* (1), *O. serpyllifolia* (2) and *N. goesingensis* Redlschlag population (3), and tested for their Ni accumulation capacities. For each strain of endophytic fungi, thirty *N. caerulescens* seedlings were inoculated in single inoculation experiments. Plants were grown in sand and vermiculite (1:2, v:v), irrigated with Hoagland solution supplemented with 150 μM $NiSO_4$. Eight week old plants were harvested for analysis. Ni concentration is presented in relation to the values measured in uninoculated control plants, statistical significance was evaluated with the *t-test* at $p \leq 0.05$ (N = 3).

glucosinolate catabolism (three and two terms respectively) and regulation of transcription. The remaining GO terms representing genes with downregulated expression in *N. caerulescens* E+ (but not in *N. goesingensis* (Flatz)) included: photosynthesis, glycine catabolic process, regulation of circadian rhythm, isoprenoid biosynthetic process, carotenoid biosynthetic process, response to UV-B, plant-type cell wall loosening, unidimensional cell growth, response to cold, chloroplast organization, DNA-templated, photorespiration, response to cytokinin, cadmium ion homeostasis, circadian rhythm, response to red light, response to abscisic acid, floral organ abscission, ergosterol biosynthetic process, brassinosteroid homeostasis, brassinosteroid, biosynthetic process, detoxification of cadmium ion, lipoate biosynthetic process. In *N. goesingensis* E+ (Flatz) (but not in *N. caerulescens*) the expression of genes involved in the following processes were downregulated: unsaturated fatty acid biosynthetic process, response to heat, protein catabolic process and long-chain fatty acid biosynthetic process. The GO terms differentially expressed in *N. caerulescens* E+ Ni+ compared to *N. caerulescens* E- Ni+ and *N. goesingensis* E+ Ni+ (Flatz) *N. goesingensis* E- Ni+ (Flatz) are shown in Table 3.

To verify transcriptomic data we quantified expression of twelve randomly selected genes with *q*PCR. The expression of 83% (10 out of 12) genes was the same using the two different methods (trend wise, variations in the magnitude of expression differences were observed (Supplementary material 1).

4. Discussion

4.1. The supportive role of endophytic fungi in adaptation of Noccaea caerulescens to Ni

Very little is known about the role of symbiotic fungi in adaptation to vegetation in serpentines. In the only available report the AM fungus *Rhizophagus intraradices* significantly increased *Berkheya coddii* growth and survival in soil enriched with high quantities of Ni. Although Ni accumulation in plant was slightly lower in inoculated plants, AMF however significantly improved biomass production by its host allowing enhanced Ni recovery from the soil (Orłowska et al., 2011; Turnau and Mesjasz-Przybyłowicz, 2003).

In our study, isolation and identification of endophytic fungi from different populations of Ni hyperaccumulators allowed us to investigate the role of these fungi in plant adaptation to Ni enriched soil. Our main objective was to obtain new insights into the role of endophytic fungi in Ni hyperaccumulation of plants from the *Noccaea* genus. The 87 strains and 40 identified OTU isolated from Ni hyperaccumulators do not exhaust the biodiversity of the endophytic fungal community inhabiting *Noccaea* and *Odontarrhena* from serpentines, but give an idea about the most common cultivable endophytic fungi inhabiting hyperaccumulators from these environments. Out of the 23 strains of fungi tested for their ability to improve plant growth in Ni enriched substrate seven had a significant impact on plant biomass production, as indicated by FW and DW. Inoculation with four different representatives of this group: *E. thlaspis*, *C. cladosporioides*, *P. cucumerina* and *Alternaria* sp. additionally led to a significant increase in Ni uptake from the substrate. This group of fungi is of particular interest for Ni phytoextraction, because: i) an improvement in biomass yield can increase overall trace element removal (as was also shown by Sessitsch et al., 2013), ii) Ni yield in aerial parts of the plant was significantly higher. Two other species: *M. verrucosa* and the second strain of *C. cladosporioides* (2) (from *N. caerulescens*) enhanced root Ni uptake without affecting biomass production by the plant and the concentration of Ni in the shoots of its host. Additionally, inoculation with *P. columnaris* and *A. africana* improved Ni accumulation in plant shoots without accelerating plant growth. These results clearly show that different species of fungi may affect different aspects of plant Ni adaptation. In natural environments, plants are inhabited by multiple species of symbiotic fungi, thus we can assume that these different microorganisms may interfere with various aspects of metal hyperaccumulation metabolism simultaneously and that the resultant accumulation phenotype is determined by the variety of plant microorganism interactions taking place within a single symbiotic

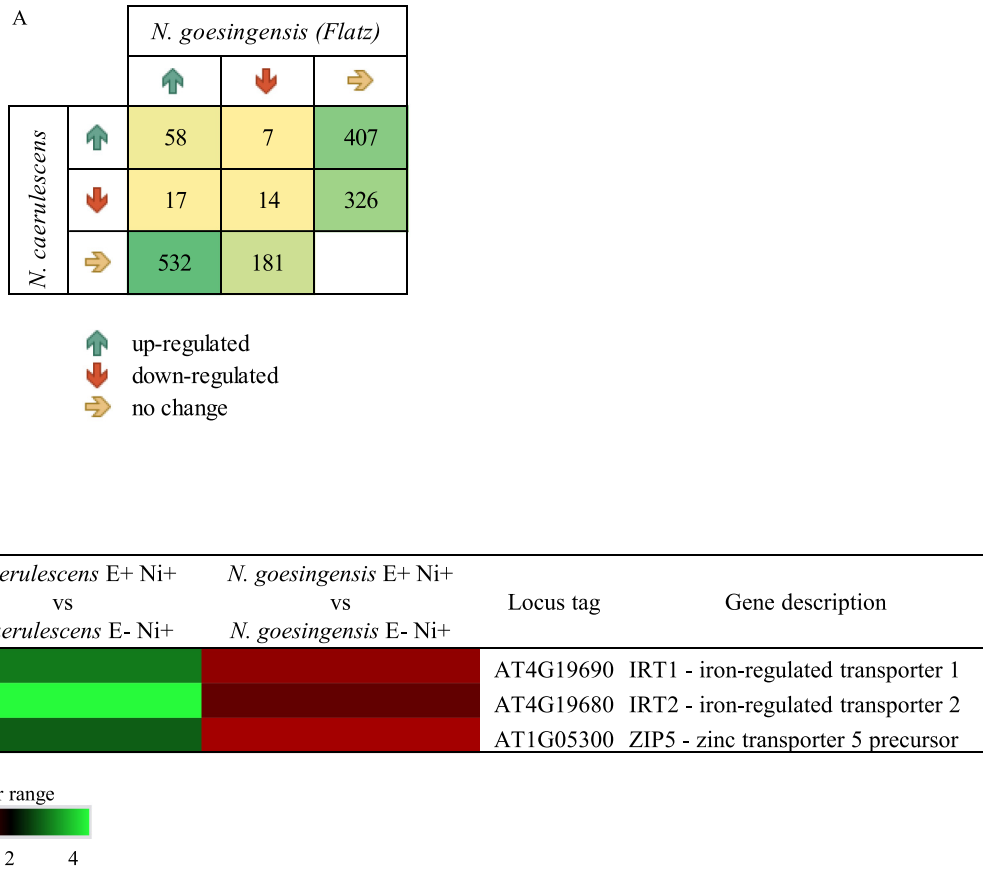


Fig. 8. Distribution of differentially expressed genes in roots of *N. caerulescens* and *N. goesingensis* (Flatz) populations inoculated with *P. columnaris* (1). For each strain of endophytic fungi, thirty *N. caerulescens* seedlings were inoculated in single inoculation experiments. Plants were grown in sand and vermiculite (1:2, v:v), irrigated with Hoagland solution supplemented with 150 μM $NiSO_4$. Eight week old plants were harvested for analysis (**A**). Comparison of the response to the inoculation within each population. Each square of the board represents common genes in both tested populations, arrow direction indicates up-, down-regulated or not-changed gene expression. The number of genes with expression unaltered in both population is not specified (empty square). Heat map representing fold change difference in the expression of genes involved in divalent metal ion transport (GO term zinc II transmembrane transport) (B). Gene expression was analyzed in *N. caerulescens* E+ Ni+ vs *N. caerulescens* E- Ni+ E+ Ni+ compared to *N. caerulescens* E+ Ni- and in *N. goesingensis* E+ Ni+ vs *N. goesingensis* E- Ni+

metaorganism/holobiont. In this study, single inoculation experiments were performed. This enabled us to dissect the effect of single endophytes on Ni hyperaccumulation of *N. caerulescens*.

To verify the stress protective role of endophytic fungi, we measured anthocyanin accumulation in shoots of plants inoculated with *P. columnaris*, *P. cucumerina*, *E. thlaspsis* and *A. africana*. All of the selected endophytes enhanced Ni uptake into plant shoots, but decreased anthocyanin production. Anthocyanins play a well-documented role in plant stress protection (reviewed in Chalker-Scott, 1999). Upregulation of their biosynthesis has been shown upon exposure to excess metals and their accumulation can be indicative of plant stress (Asad et al., 2015; Hawrylak-Nowak, 2008). In all of the here examined plants the shoot concentration of these compounds was significantly lower compared to not inoculated control, although plants accumulated higher quantities of Ni in their shoots; this indicates that the presence of the symbiotic microorganism limited plant stress.

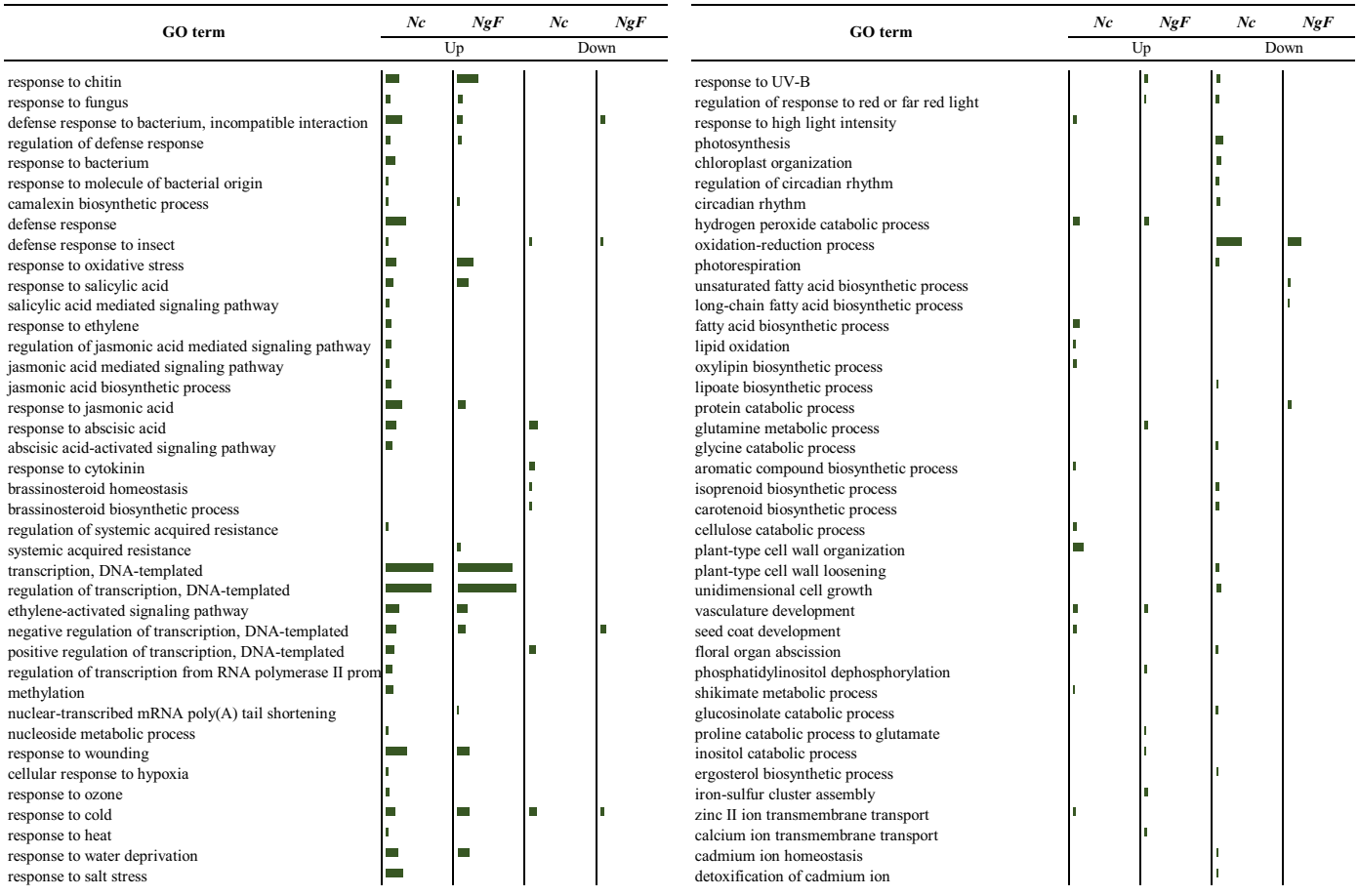
4.2. Phomopsis columnaris – the most frequently isolated species of endophytic fungi from hyperaccumulators

The fungus species most frequently isolated from hyperaccumulator plants, independently of the plant origin, was *Phomopsis columnaris*. It was isolated from all three serpentine ecosystems investigated in this study. We identified this species in plants inhabiting other metalliferous environments (data not shown), and it was also found in *Sceletium tortuosum* (Manganyi et al., 2018), *Myrtus communis* (Vaz et al., 2012) and olive tree *Olea europaea* in Northeast Portugal, where it represented 47% of the total isolates (Martins et al., 2016). Inoculation with *P. columnaris* has been shown to be beneficial for *Sorghum vulgare* (Vaz et al., 2012), however, in another study it caused twig dieback of *Vaccinium vitis-idaea* (Farr et al., 2002). Interestingly, our attempts to co-cultivate *Arabidopsis thaliana* with *Phomopsis columnaris* (strain isolated from *N. caerulescens*) turned out lethal for the host (unpublished data). To verify metal tolerance of *P. columnaris* used in this study we measured the tolerance index of this fungus to Ni and other potentially toxic metals. This allowed us to verify the persistence and usefulness of this strain during application to the soil and also to verify whether adaptation to metal toxicity of the fungus is limited to the metal species that it was exposed to in its natural environment. The strain turned out to be highly resistant to metals and its tolerance was not limited to Ni, but in comparison to another *P. columnaris* strain, isolated from *Arabidopsis arenosa* from a post mining waste dump (exposed to extremely high concentrations of Zn, Fe and Cd), its resistance was significantly lower. This indicates that fungi metal tolerance depends on mechanisms that are not metal species specific (or has low metal specificity); that adaptation to metal toxicity is related to the activation of general metal stress related mechanisms rather than involving mechanisms related to a particular metal species. Additionally, these results confirm the role of adaptation in fungi metal tolerance.

4.3. Variability of the response of different hyperaccumulators to different strains of Phomopsis columnaris

In the majority of bioaugmentation studies, the effects of re-inoculating host plants with their associated isolates have been evaluated (Cabello-Conejo et al., 2014; Durand et al., 2016). However, the specificity of these plant-microbe (tested for bacteria and AMF)

Table 3
The enriched molecular function GO terms were searched among the differentially expressed genes in root of the *N. caerulescens* (Nc) and *N. goesingensis* (Flatz population, NgF) populations inoculated with *P. columnaris* (1) upon Ni treatment using DAVID functional annotation tool.



combinations is not always clear, and some inoculants have been shown to have beneficial effects over a wide range of plant hosts (Becerra-Castro et al., 2012; Ma et al., 2013). In other cases the plant-microorganism interaction can be neutral, or have a negative impact on vegetation (reviewed in Mandyam and Jumpponen, 2015; Schulz and Boyle, 2005). Balancing growth, defense and energy distribution between the two symbiotic partners is of great significance and seems to determine beneficial symbiosis versus disease. Various fungi species may act as parasites or mutualists depending on host species, stage of ontogenesis, also *exo*- or endogenous cues may disturb the symbiotic equilibrium causing endophytes to become parasites and vice-versa. Thus, the plant-*P. columnaris* symbiosis may be an example of "conditional mutualism" (Rodriguez et al., 2009). In fact, disease may be regarded as an unbalanced state of symbiosis. In our experiments, *Noccaea* and *Odontarrhena* plants inoculated with *P. columnaris* (independently of the fungus strain) did not exhibit any signs of pathogenesis. *P. columnaris* was isolated from three distinct plant species inhabiting three different serpentine ecosystems. In the previously described experiment, we showed that symbiosis with *P. columnaris* resulted in increased accumulation of Ni its native host *N. caerulescens* without negatively affecting the plants performance. The remaining two strains (one from *N. goesingensis* and the other from *O. serpyllifolia*) did not have any effect on Ni accumulation of *N. caerulescens*. When we inoculated *N. goesingensis* (Redlschlag) with its native strain of *P. columnaris*, it increased its ability to take up Ni, but inoculation with the two non-native *Phomopsis* strains had no effect on Ni accumulation by the plant. In both *Noccaeae* Ni adapted species only symbiosis with its native endophyte resulted in improved ability to accumulate higher quantities of Ni. This indicates that the phenotype is strain specific and probably requires long-term adaptation of the plant and microorganism. The Ni non-adapted *N. goesingensis* ecotype (Flatz) responded only to inoculation with the *P. columnaris* strain from *N. caerulescens*, it accumulated significantly more Ni, but metal concentrations in the shoot did not reach concentrations found in hyperaccumulators and the distribution of the metal (root and shoot) did not follow a typical hyperaccumulator type pattern. This suggests that the symbiotic microorganism can have a positive effect on Ni accumulation of its host plant, but enhances the hyperaccumulator phenotype only in hyperaccumulators.

Metal hyperaccumulators have developed a unique ability to accumulate high concentrations of potentially toxic metals. The physiology of hyperaccumulation relies on the following processes: i) enhanced metal influx across root cell plasma membranes (Pence et al., 2000); ii) reduced metal sequestration in root vacuoles (Lasat et al., 1998); iii) increased metal xylem loading and root to shoot translocation (Papoyan and Kochian, 2004; Hanikenne et al., 2008) and iv) stimulated metal influx across leaf cell plasma membranes and sequestration in leaf vacuoles (Küpper et al., 2001, 2004).

According to Verbruggen et al. (2009) metal hyperaccumulation relies on processes that are not species-specific or novel, but rather differently expressed and regulated, compared with non-hyperaccumulator species. Previous studies indicate that hyperaccumulators can only reach their full capacity to accumulate metals in the presence of indigenous rhizosphere microbiota (Becerra-Castro et al., 2013; Sessitsch et al., 2013). However, very little is known about the role of the plants inherent microbiota in this process. Thus, we hypothesized that the fungal symbiont dependent Ni accumulation phenotype relies on alterations in regulation of processes associated with hyperaccumulation physiology. Upon inoculation with *P. columnaris*, *N. caerulescens* and

N. goesingesis (Redlschlag) accumulated 2-fold more Ni in the shoot, exceeding 1000 mg·kg^{-1}, whereas E+ *N. goesingesis* (Flatz) was able to take up significantly more Ni into its roots, but significantly less (ca. 60% in roots) compared to E+ *N. caerulescens* and *N. goesingesis* (Redlschlag). The *N. goesingensis* (Flatz) E+ shoot concentration of Ni reached 450 mg·kg^{-1}. These results clearly indicate that the symbiotic microorganism enhanced hyperaccumulator traits in the Ni adapted plants/ecotypes (Ni concentration over 1000 mg·kg^{-1}, highly significant shoot accumulation of up took Ni), whereas in the not Ni adapted *N. goesingensis* it improved Ni uptake, but it did not induce typical hyperaccumulator traits.

4.4. Inoculation with Phomopsis columnaris and the resultant increase in Ni uptake by the plant activates specific gene networks

To identify gene networks affected by the symbiotic microorganism and potentially involved in enhanced Ni uptake we compared the response of the transcriptome of *Noccaea caerulescens* E+ Ni+ with *N. caerulescens* E- Ni+ and *N. goesingensis* (Flatz) E+ Ni+ vs *N. goesingensis* (Flatz) E- Ni+. The number genes with up- or down-regulated expression in both populations were fairly low (the expression of 14 genes was down-regulated, whereas the expression of 58 genes was up-regulated) and the majority of genes were involved in: plant defense response (against biotic and abiotic factors), phosphate homeostasis and root and vasculature development. All of these processes are typically altered in plants during symbiotic interactions (reviewed in Domka et al., 2019a, 2019b). The majority of the genes differently regulated in the two *Noccaea* species differed in both species after inoculation, suggesting differential response of the two plants to the fungus. Interestingly, the majority of these genes were classified into the same functional categories in both species, suggesting that similar biological processes are affected by the fungus, but different aspects of these processes are regulated depending on the plant species. Out of the differently expressed genes, the best suited to explain Ni accumulation phenotype of E+ *N. caerulescens* where genes belonging to the GO term "Zn II ion transmembrane transport". The expression of three genes: *IRT1* (*IRON REGULATED TRANSPORTER 1*), *IRT2* (*IRON REGULATED 2*) and *ZIP5* (*ZINC TRANSPORTER 5 PRECURSOR*) was upregulated in *N. caerulescens* E+ Ni + compared to *N. caerulescens* E+ Ni-, whereas no changes in their expression were found in *N. goesingensis* (Flatz) E+ Ni+ vs *N. goesingensis* (Flatz) E- Ni+.

IRT1 and IRT2 were identified as membrane bound Fe transporters (Fe uptake and radial transport in plant roots respectively) with varying affinity to other divalent metal cations (Korshunova et al., 1999; Rogers et al., 2000; Vert et al., 2001) including Ni (Nishida et al., 2011). ZIP5 was shown to be involved in metal ion (Zn) transmembrane transport. No reports are confirming its role in Ni transport available, but we cannot exclude its role in Ni homeostasis. An upregulation of expression of genes encoding IRT1 and IRT2 was also found in the Ni adapted ecotype of *N. goesingensis* (Redlschlag) upon exposure to Ni, whereas the expression of these two genes was down regulated in the not adapted ecotype of *N. goesingensis* (Flatz), suggesting their role in Ni hyperaccumulation (Domka et al., 2020). Additionally, in a metal adapted accession of *N. caerulescens* (Halimaa et al., 2014) and a Zn hyperaccumulating population of *A. halleri* (Schvartzman et al., 2018), IRT1, IRT2 encoding genes were suggested to be involved in metal, including Ni hyperaccumulation. The mechanism responsible for Ni xylem loading has not been identified to date, however available studies indicate the possible involvement of Zn or/and Fe transport system (Assuncao et al., 2001; Ghasemi et al., 2009). IRT1 was suggested to be responsible for Mn vasculature transport/xylem loading in *Hordeum vulgare*, we therefore cannot exclude its possible role in Ni xylem loading in *Noccaea* (Long et al., 2018). The IRT1 protein was found to be expressed mainly in root epidermal cells of *A. thaliana*, where it participates in metal uptake from the soil (Dubeaux et al., 2018). The IRT1 localization in roots of plants from the *Noccaea* genus has not been investigated up to date, therefore we cannot exclude the possible role of this transporter in Ni uptake and distribution. Further studies are required to confirm this.

5. Conclusions

The plant/rhizosphere microbiome plays a significant role in plant Ni bioaccumulation. Several authors reported that by reshaping the plants microbiome we can further enhance the efficiency of phytoextraction and phytomining. However, the efficiency of microorganisms that potentially could be used as bioinoculants is dependent on a complex array of interacting factors, one of them being plant-microbe compatibility. As shown here different strains of endophytic fungi affect metal hyperaccumulation by either accelerating plant growth or by activating Ni uptake by the plant. Additionally, all of the beneficial microorganism tested in this study protected the plants from excess Ni stress. These results show that endophytic fungi play various, essential roles in the process of Ni hyperaccumulation. In this study, we focused our attention on the role of endophytic fungi in Ni accumulation. However, an important feature of the inoculated plants was their enhanced adaptation to metal toxicity. The increase in Ni accumulation of E+ plants, independently of plant species, co-occurred with upregulation of genes involved in plant defense. The transcriptomic data published here can be a good starting point in elucidating the mechanisms of symbiotic fungi activation of plant defense. The altered expression of particular gene networks suggests that the enhancement of the hyperaccumulator phenotype by the endophytic fungus may result from activation of Ni transport from the rhizosphere to and within plant roots with parallel activation of plant defense mechanisms.

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CRediT authorship contribution statement

Rafał Ważny: Conceptualization, Investigation, Methodology, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Piotr Rozpądek:** Conceptualization, Investigation, Methodology, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. **Agnieszka Domka:** Conceptualization, Investigation, Methodology, Formal analysis, Visualization, Writing – review & editing. **Roman J. Jędrzejczyk:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Michał Nosek:** Investigation. **Magdalena Hubalewska-Mazgaj:** Investigation. **Irene Lichtscheidl:** Investigation, Writing – review & editing, Funding acquisition. **Petra Kidd:** Investigation. **Katarzyna Turnau:** Conceptualization, Investigation, Methodology, Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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